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ANTE- AND POSTMORTEM DIAGNOSTIC TECHNIQUES FOR ANTHRAX: RETHINKING PATHOGEN EXPOSURE AND THE GEOGRAPHIC EXTENT OF THE DISEASE IN WILDLIFE

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ABSTRACT: Although antemortem approaches in wildlife disease surveillance are common for most zoonoses, they have been used infrequently in anthrax surveillance. Classically, anthrax is considered a disease with extremely high mortality. This is because anthrax outbreaks are often detected ex post facto through wildlife or livestock fatalities or spillover transmission to humans. As a result, the natural prevalence of anthrax infection in animal populations is largely unknown. However, in the past 20 yr, antemortem serologic surveillance in wildlife has indicated that not all species exposed succumb to infection, and anthrax exposure may be more widespread than originally appreciated. These studies brought about a multitude of new questions, many of which can be addressed by increased antemortem serologic surveillance in wildlife populations. To fully understand anthrax transmission dynamics and geographic extent, it is important to identify exposure in wildlife hosts and associated factors and, in turn, understand how these influences may drive environmental reservoir dynamics and concurrent disease risk in livestock and humans. Here we review our current understanding of the serologic response to anthrax among wildlife hosts and serologic diagnostic assays used to augment traditional postmortem anthrax surveillance strategies. We also provide recommendations for the use of serology and sentinel species surveillance approaches in anthrax research and management.

Key words: Anthrax, antibody prevalence, *Bacillus anthracis*, exposure, sentinel species, serology, wildlife disease surveillance, zoonosis.

INTRODUCTION

Recently attention has refocused on wildlife as sentinels for zoonoses and as reservoirs for (re)emerging diseases (Halliday et al., 2007; Alexander et al., 2012; Jobbins et al., 2013). Although many research and public health programs investigating zoonoses routinely utilize serology and other antemortem approaches, serology is less often employed for zoonoses with rapid disease progression leading to death. One such example is anthrax, a highly lethal and widespread zoonosis, for which regular surveillance is uncommon in wildlife and often only occurs in response to a specific outbreak during a limited time.

Anthrax, caused by the spore-forming bacterium *Bacillus anthracis*, is a disease of concern nearly worldwide (Hugh-Jones and Blackburn, 2009). Despite the zoonotic risk, the disease remains underdiagnosed, underreported, and undervalued as a public and wildlife health concern (Fasanella et al., 2010) on nearly every continent with human cases being common in agrarian or developing countries (Turnbull et al., 2008). Many wildlife populations experience regular epizootics, and *B. anthracis* can infect a wide range of mammalian species (Hugh-Jones and De Vos, 2002).

Transmission pathways, though poorly understood, include cutaneous contact, percutaneous infection from biting flies, ingestion, or inhalation of *B. anthracis* spores (Alexander et al., 2012). Within a carcass, *B. anthracis* remains in a vegetative state until exposed to oxygen, usually by scavengers or humans disturbing or opening an infected carcass. *Bacillus*

anthracis spores are taken up by macrophages and enter regional lymph nodes, germinate, and replicate. Tripartite toxin, consisting of protective antigen (PA), edema factor (EF), and lethal factor (LF), is released into the lymph and eventually into the bloodstream. A PA-EF toxin complex is responsible for the severe edema related to anthrax infection in its hosts, while PA-LF complex is internalized and kills cells (Turnbull et al., 2008).

In susceptible herbivores, anthrax is characterized by a 36–72 hr incubation period, during which clinical signs often include nonspecific behavioral changes, followed by a systemic acute phase resulting in sudden death (Turnbull et al., 2008). Anthrax outbreaks are generally first detected after several animals die (Turnbull et al., 2008). Data on the infectious dose, time to death, and exposure routes are limited (Hugh-Jones and Blackburn, 2009). Because of the swift, lethal progression of infection in susceptible animals, anthrax was historically considered a universally severe disease with few survivors. Starting in 1989, serologic testing indicated wildlife are regularly exposed (Turnbull et al., 1989, 1992), and some exposed animals appear to recover (Rijks, 1999). These initial investigations raise important new questions about wildlife exposure, many of which could be addressed through increased antemortem serologic surveillance. We discuss the state of knowledge regarding anthrax susceptibility and serologic responses in wildlife, identify questions emerging from previous studies, and discuss future directions for anthrax wildlife research. We briefly overview classical anthrax surveillance, review antemortem serologic approaches, and include recommendations for the use of serology and sentinel species in anthrax surveillance.

ANTHRAX EXPOSURE AND SURVIVAL OF INFECTION BY WILDLIFE

Classical anthrax surveillance in wildlife entails discovery of suspect carcasses and bacterial culture to confirm *B. anthracis* infection. However, using opportunistic mortality data, or wildlife carcass discovery rates in areas of high-human traffic (e.g., national park roads), leads to a skewed representation of the species potentially infected (Bellan et al., 2013). Small-bodied wildlife (especially carnivores and some herbivores, e.g., white-tailed deer, *Odocoileus virginianus*; Blackburn and Goodin, 2013) are more difficult to detect than large-bodied herbivores. Therefore, postmortem bacterial culture is unlikely to identify the full host range, numbers affected, or variation in survival rates.

From the outset, researchers observed the frequency of herbivore carcass recovery during wildlife (and livestock) outbreaks exceeded the number of carnivore carcasses. There is often little evidence of predators or scavengers dying from anthrax or with anthrax-related pathology or illness (Hampson et al., 2011). Serologic investigations on African wildlife support these postmortem observations. Antibody prevalence is higher in carnivores and omnivores (63–100%) than in herbivores in the same regions (0–46%; Table 1). Carnivores from Namibia were found to exhibit high antibody titers to all three components of anthrax toxin in contrast to herbivores (Turnbull et al., 1989, 1992). These data, coupled with the mortality imbalance between carnivores/omnivores and herbivores, suggest carnivores and omnivores are more resistant than herbivores. Currently it is unknown whether this resistance results from differences in dosage, physiology/immunology, behavior, transmission route, or some combination of these factors. Herbivores are most likely infected through ingestion during grazing/browsing (Blackburn et al., 2010), percutaneous exposure by biting flies (Blackburn, 2010), or possibly spore inhalation. In carnivores/omnivores, infection likely occurs from eating infected carcasses. Clinical signs or death in wild dogs (*Lycaon pictus*; Creel et al., 1995), domestic dogs

TABLE 1. Summary of published antibody to anthrax protective antigen (PA) prevalence data in wildlife species and their feeding and social behavior.

Common name	Family	Genus	Species	Feeding[a]	Feeding behavior
Cheetah	Felidae	*Acinonyx*	*jubatus*	Carnivore	Predator
Lion		*Panthera*	*leo*	Carnivore	Predator/ scavenger
Hyena, Spotted	Hyaenidae	*Crocuta*	*crocuta*	Carnivore/ opp. omnivore	Predator/ scavenger
Hyena, Brown		*Hyaena*	*brunnea*	Carnivore/ opp. omnivore	Predator/ scavenger
Jackal, Black-backed	Canidae	*Canis*	*mesomelas*	Carnivore/ opp. omnivore	Predator/ scavenger
Dog, Domestic		*Canis*	*familiarus*	Carnivore/ omnivore	Predator
Vulture, Cape	Accipitridae/ subfamily Aegypiinae	*Gyps*	*coprotheres*	Carnivore	Scavenger
Vulture, White-faced		*Gyps*	*africanus*	Carnivore	Scavenger
Vulture, Lappet-faced		*Aegypius*	*tracheliotus*	Carnivore	Scavenger
Pigs, experimentally infected by feed	Suidae	*Sus*	*scrofa domesticus*	Omnivore	Forager
Pigs, retested from Edginton (1990)					
Springbok	Bovidae/ subfamily Antilopinae	*Antidorcas*	*marsupialis*	Herbivore	Browser/ grazer
Bison, Wood	Bovidae/ subfamily Bovinae	*Bison*	*bison athabascae*	Herbivore	Grazer
Buffalo, African	Bovidae/ subfamily Bovinae	*Syncerus*	*caffer*	Herbivore	Grazer
Buffalo, African					
Cattle (dairy herd)		*Bos*	*taurus*	Herbivore	Grazer
Wildebeest	Bovidae/ subfamily Alcelaphinae	*Connochaetes*	*taurinus*	Herbivore	Grazer
Elephant	Elephantidae	*Loxodonta*	*africana*	Herbivore	Browser/ grazer
Giraffe	Giraffidae	*Giraffa*	*camelopardalis*	Herbivore	Browser
Deer, Red (elk)	Cervidae/ subfamily Cervinae	*Cervus*	*elpahus*	Herbivore	Grazer/ Browser

TABLE 1. Extended.

Behavior	Location[b]	Positive for antibody to PA % (*n*)	ELISA[c]	Reference
Solitary	Jwaneng, Botswana	4, 6 (16;23)[d]	Turnbull et al., 2004	Good et al., 2008
Social	Etosha NP, Namibia	97 (31)	Turnbull et al., 1992	Turnbull et al.,1992
	Desert region, Namibia	0 (3)[e]	Turnbull et al., 1992	Turnbull et al.,1992
	Pretoria Zoo, South Africa	0 (2)	Turnbull et al., 1992	Turnbull et al., 1992
	Serengeti NP, Tanzania	90 (263)	Immunetics	Lembo et al., 2011
	Ngorongoro Crater, Tanzania	57 (23)	Immunetics	Lembo et al., 2011
	Malilangwe Wildlife Preserve, Zimbabwe	17 (12) pre-outbreak; 92(13) post-outbreak	Turnbull et al., 1992	Clegg et al., 2007
Social	Etosha NP, Namibia	100 (10)	Turnbull et al., 1992	Turnbull et al., 1992
	Serengeti NP, Tanzania	87 (53)	Immunetics	Lembo et al., 2011
Less social than spotted hyena	Adjacent farm to Etosha NP, Namibia	0 (1)[e]	Turnbull et al., 1992	Turnbull et al., 1992
Solitary, pairs, or small groups	Etosha NP, Namibia	95 (86)	Turnbull et al., 2004	Bellan et al., 2012
	Etosha NP, Namibia	100 (8)[f]	Turnbull et al., 1992	Turnbull et al., 1992
	Desert region, Namibia	0 (4)[f]	Turnbull et al., 1992	Turnbull et al., 1992
Social	Serengeti NP, Tanzania	25 (314)	Immunetics	Hampson et al., 2011
Social	Etosha NP, Namibia	67 (6)	Turnbull et al., 2010	Turnbull et al., 2010
Social	Captive, various regions of South Africa	0 (15)	Turnbull et al., 2010	Turnbull et al., 2010
Social	Etosha NP, Namibia	60 (10)	Turnbull et al., 2010	Turnbull et al., 2010
Social	Etosha NP, Namibia	33 (3)	Turnbull et al., 2010	Turnbull et al., 2010
Social	Livestock, experiment	88 (50)	Turnbull et al., 1989	Redmond et al., 1997
	Livestock, Wales	4 (26)	Turnbull et al., 1989	Redmond et al., 1997
Social, herds (same-sex)	Etosha NP, Namibia	15 (20)	Turnbull et al., 1992	Turnbull et al., 1992
Social	Peace River, Canada	85 (20) preepizootic bison	Rijks et al., 1999	Rijks et al., 1999
Social, same-sex herd and family herd	Serengeti NP, Tanzania	46 (28)	Immunetics	Lembo et al., 2011
	Ngorongoro Crater, Tanzania	14 (21)	Immunetics	Lembo et al., 2011
Social, herd	West Country, England	5 (20)	Turnbull et al., 1992	Turnbull et al., 1992
	Midlands, England, July–August 1990	77 (13)	Turnbull et al., 1992	Turnbull et al., 1992
	Midlands, England, July–August 1990	27 (22)	Turnbull et al., 1992	Turnbull et al., 1992
	Midlands, England, September 1990	0.7 (30)	Turnbull et al., 1992	Turnbull et al., 1992
Social, herd	Etosha NP, Namibia	0 (1)[g]	Turnbull et al., 1992	Turnbull et al., 1992
	Serengeti NP, Tanzania	19 (36)	Immunetics	Lembo et al., 2011
	Ngorongoro Crater, Tanzania	4 (23)	Immunetics	Lembo et al., 2011
Social	Etosha NP, Namibia	0 (28)[g]	Turnbull et al., 1992	Turnbull et al., 1992
Not very social	Etosha NP, Namibia	5 (19)[g]	Turnbull et al., 1992	Turnbull et al., 1992
Social, herds (same-sex)	Pollino NP, Italy	22 (27)	Unpublished	Fansanella et al., 2007

TABLE 1. Extended Continued.

Common name	Family	Genus	Species	Feeding[a]	Feeding behavior
Deer, White-tailed	Cervidae/subfamily Capreolinae	*Odocoileus*	*virginianus*	Herbivore	Browser
Rhino	Rhinocerotidae	*Diceros*	*bicornis*	Herbivore	Browser
Zebra	Equidae	*Equus*	*burchellii*	Herbivore	Grazer

[a] Opp. = opportunistic.

[b] NP = National Park.

[c] ELISA = enzyme-linked immunosorbent assay.

[d] Twenty-three wild-caught cheetahs; 16 of 23 had access to the game reserve where anthrax outbreak occurred.

[e] Used as negative control for Etosha counterparts.

[f] No negative control available during assay run.

[g] No positive control available during assay run.

[h] No difference in ELISA results between deer from anthrax endemic and nonendemic areas and no difference in ELISA results after an epizootic and 4–5 yr later.

[i] Probably vaccinated.

(*Canis familiaris*; Depaillat, 1960; Patra et al., 1998), and captive cheetahs (*Acinonyx jubatus;* Good et al., 2005) were linked to ingestion of infected meat. These apparent differences in exposure routes and magnitude may influence the pathology and allow differential levels of resistance among species. Additionally, predators and scavengers often have lower stomach pH than ruminants and other herbivores, which may inactivate ingested spores or vegetative cells (Turnbull et al., 2010).

Are all herbivores equally susceptible and carnivores equally resistant?

Generally herbivores appear more susceptible to infection than carnivores. At the same time, serosurveillance data reveal gradations of susceptibility or resistance among species. For example, in northern Canada, a significant proportion of post-anthrax epizootic wood bison (*Bison bison athabascae*, 70%, 14/20) had intermediate to high antibody titers to all three components of anthrax toxin in comparison to the pre-epizootic population (Rijks, 1999). The author concluded that bison surviving epizootics developed a protective immune response represented by high antibody titers. In Tanzania, African buffalo (*Syncerus caffer*) had a higher antibody prevalence than other herbivores (46% and 14% in Serengeti National Park and Ngorongoro Crater, respectively; Table 1; Lembo et al., 2011). In contrast, zebras from multiple areas appeared to be highly susceptible to anthrax infection with 0/119 animals surveyed in Tanzania and Namibia being antibody positive (Turnbull et al., 1989; Lembo et al., 2011; Table 1). However, recent evidence from Etosha National Park suggests that some zebra do seroconvert (Cizauskas, 2013), indicating that there may regional differences in herbivore susceptibility.

Susceptibility may differ among carnivores as well. Lions (*Panthera leo*) often exhibit antibody prevalence, as high as 97% in some populations (Table 1). In contrast, wild cheetahs had low antibody prevalence (4%; Good et al., 2008). This suggests lions are exposed more often or are more likely to survive exposure than cheetahs. Cheetahs appear to be more

TABLE 1. Extended Continued.

Behavior	Location[b]	Positive for antibody to PA % (*n*)	ELISA[c]	Reference
Social, mainly same-sex groups during outbreak period	Woodson Ranch, Carta Valley, Texas, USA	Anthrax endemic $n=45$; nonendemic $n=70$[h]	Peterson et al., 1993	Peterson et al., 1993
Mostly solitary	Etosha NP, Namibia	14 (29)[g,i]	Turnbull et al., 1992	Turnbull et al., 1992
Social, herd	Serengeti NP, Tanzania	0 (74)	Immunetics	Lembo et al., 2011
	Ngorongoro Crater, Tanzania	0 (11)	Immunetics	Lembo et al., 2011
	Etosha NP, Namibia	0 (34)	Turnbull et al., 1992	Turnbull et al., 1992

sensitive to infection, as cheetah anthrax mortalities are more frequent during outbreaks (Hampson et al., 2011; Bellan et al., 2012). Also, captive cheetahs developed rapid disease progression and death when fed anthrax-contaminated meat (Good et al., 2005).

Are predators and scavengers truly infected?

High antibody prevalence in predators and scavengers could result from surviving infection or from absorbing PA (and other toxin components) across the mucous membrane of the gut. Once in the host circulatory system, PA would act as a foreign antigen and stimulate antibody formation (Turnbull et al., 2010). Both situations would result in elevated titers to the toxin molecules. Serology for capsule antigen could potentially distinguish between absorption and active infection. There are no reported infection experiments in carnivores. Some LD_{50} studies from the 1950–1960s include omnivorous laboratory model and domestic species (Lincoln et al., 1967; reviewed by Watson and Keir, 1994) but had no serologic data. Recently, in an experimental infection study in an omnivorous scavenger (domestic pigs; *Sus scrofa*), 66% of pigs developed clinical signs of anthrax infection. Most seroconverted at day 21 postinoculation; two died, and the rest cleared infection (Redmond et al., 1997). As most showed clinical signs, but did not succumb to infection, the pigs were considered resistant to disease.

Is anthrax sex- and age-biased?

Current knowledge of gender bias in anthrax infection is limited to mortality records. During a 2005 outbreak in Kenya, Muoria et al. (2007) reported male-biased mortality for Grevy's zebras (*Equus grevyi*) and female-biased mortality for plains zebras (*Equus burchelli*) without reporting the sex ratios for the species in the region. Sex-biased mortality was also reported in multispecies outbreaks in Namibia (Lindeque and Turnbull, 1994), Ethiopia (Shiferaw et al., 2002), and Zimbabwe (Clegg et al., 2007). In white-tailed deer anthrax epizootics, buck mortalities exceeded that of does (Blackburn, 2006). During a large epizootic in southwestern Montana, USA, in 2008, bull plains bison (*Bison bison bison*) mortality was ~27%, while cow mortality was ~8% (Blackburn, unpubl. data). Additionally, at least 45 bull elk (*Cervus elaphus*) were found dead around the bison focus, while cow elk carcasses were absent despite extensive aerial and ground searches (Blackburn, unpubl. data). Gender and age ratios have not been reported as part of serologic surveys, and such data may determine if exposure is as gender biased as mortality.

CAVEATS AND FUTURE DIRECTIONS

Serologic investigations to assess prevalence of anthrax antibodies have primarily focused on wildlife from African national parks (Turnbull et al., 1992; Hampson et al., 2011; Lembo et al., 2011; Bellan et al.,

2012). Also, there have been three times more serologic tests reported in the literature for African carnivores ($n=880$) than herbivores ($n=282$), and authors report possible sensitivity issues for serologic assays in several African herbivore species (Table 1; Turnbull et al., 1992). More simultaneous regional serologic investigation of herbivores, omnivores, and carnivores using well-matched serologic assays or other methods engineered for cross-species investigation are needed. Use of antemortem surveillance measures, especially in tandem with postmortem investigations, would identify which species, genders, and individuals are being exposed, which are succumbing to infection, and help determine the relationship between exposure and survival.

There is a paucity of knowledge regarding anthrax transmission routes and pathology in wildlife. The majority of data available on LD_{50} and routes of anthrax infection are from laboratory animal models and livestock (Lincoln et al., 1967; Watson and Keir, 1994). Such data do not exist for wildlife species beyond anecdotes in the literature (Watson and Keir, 1994). There is documentation of quantitative septicemial dynamics of *B. anthracis* in laboratory models and some domestic animals (Lincoln et al., 1967), but no published records relating stages of bacteremia and antibody titers in laboratory, livestock, or wildlife species. The lack of controlled data makes it difficult to interpret serologic results, particularly in cases where there is doubt as to whether the animal is truly infected (e.g., predators, scavengers) or where there are troublesome antibody results, as in vultures (Accipitridae; Turnbull et al., 2010). Additionally, not all species seroconvert similarly (Turnbull et al., 1992; Rijks, 1999; Lembo et al., 2011). Although serologic data can elucidate exposure patterns, one cannot distinguish between high exposure with recovery and low dose exposure with seroconversion. It is impossible to differentiate the two without concurrently determining infection patterns by measuring antigen or bacterial loads or by controlled laboratory infection studies. Although laboratory experiments exploring anthrax dynamics in wildlife species would be ideal, they are unlikely. Increased serologic surveillance of susceptible wildlife populations coupled with bacterial culture, antigen detection, or molecular methods will allow better interpretation of antibody levels and clarify anthrax disease dynamics in wildlife. Subsequently, these data will clarify background occurrence and enable determination of the baseline of anthrax exposure. These data should allow better outbreak prediction and control strategies.

LOCATING ANTHRAX FOCI AND DETERMINING GEOGRAPHIC EXTENT OF DISEASE RISK

An important question about anthrax is determining where the disease occurs on the landscape and the geographic limits of a known focus. To date, such estimates are based on modeling mortality data (Blackburn et al., 2007; Joyner et al., 2010; Mullins et al., 2011; Kracalik et al., 2012) and are known to underestimate the intensity of outbreaks (Bellan et al., 2013). Mortality data may also underestimate the geographic extent of outbreaks. Serologic screening would be useful in determining background exposure across the landscape and would improve estimates of the pathogen's extent within enzootic zones. Since detectable antibody titers appear to be limited to ~1 yr (in equids, as little as 6 mo; Cizauskas, 2013), serologic screening would also indicate when animals are being exposed in the absence of major mortality events.

In addition to providing a snapshot of anthrax exposure of Tanzanian wildlife, recent studies also focused on determining whether antibody prevalence in surveyed animals could be linked to location and ecologic variables (Hampson et al., 2011; Lembo et al., 2011). African buffalo antibody prevalence had a significant

relationship with soil alkalinity (Hampson et al., 2011). Serosurveillance of carnivores and omnivores often help identify disease risk zones. For example, higher antibody titers in lions were linked to recent exposure and were associated with anthrax foci in Namibia (Turnbull et al., 1989). Also, seroconversion in domestic dogs in the Serengeti was associated with areas of anthrax-related livestock deaths (Lembo et al., 2011). Location was a significant predictor of whether a dog would be antibody positive (Hampson et al., 2011). The authors suggested using domestic dogs as sentinels to assess human anthrax risk. However, for wildlife risk, the use of domestic dogs is not ideal, as they are often not found in national parks and have a limited home range in comparison to other carnivores, such as vultures.

A traditionally effective and widely used method to discover animal carcasses is to follow vultures to carcasses sites (Bellan et al., 2013; Blackburn and Goodin, 2013). As PA antibody detection in African vultures mirrored areas of anthrax outbreaks (Turnbull et al., 2010), the authors suggested using serologic testing of vultures to determine anthrax risk zones over a large geographic range. This study indicated the usefulness of vulture serology as an efficient surveillance method to determine the geographic limits of anthrax in Africa and other anthrax-prone regions. Expanding surveillance of vultures in multiple environments, as well as to other scavengers and predators, could provide a more complete estimate of the extent of regional anthrax zones. Even if scavengers do not experience true anthrax infection, they may still have utility as predictors of geographic risk zones.

SURVEILLANCE

Bacterial culture remains the gold standard for confirmation of *B. anthracis*, although PCR is also a robust diagnostic tool when appropriate markers are selected (Easterday et al., 2005; Okinaka et al., 2006). Much of our understanding of the spatial distribution and ecology of anthrax is based on data obtained from using these assessment tools on postmortem samples. Mortality data are often used to estimate outbreak severity, timing, and extent. This may be inaccurate, because carcasses may be difficult to find or missed entirely during outbreak surveillance (Bellan et al., 2013), leading to underestimates of the occurrence and spatial extent of outbreaks (Rijks, 1999; Fasanella et al., 2010; Lembo et al., 2011). Antemortem screening methods are needed in combination with current postmortem approaches to provide a clearer picture of exposure and survival among affected host populations.

There are two main anthrax antemortem serologic surveillance methods in use: enzyme-linked immunosorbent assays (ELISAs) and lateral flow immunochromatographic assays (LFIAs; see Glossary). Anthrax ELISAs are typically designed to detect antibody to a component of anthrax toxin in serum. Anthrax LFIAs can be used to detect either PA in blood or spores from environmental samples (see advantages and disadvantages of ELISAs and LFIAs, Table 2).

Enzyme-linked immunosorbent assay

Advantages: ELISAs are relatively inexpensive, high-throughput disease surveillance tests with low complexity. They can measure immunoglobulin (Ig; see Glossary) titers and differentiate Ig types (such as IgM and IgG) or subtypes ($IgG_{1,2,3,4}$). These data may be used to extrapolate the time course of infection, especially if serial samples are available. Determination of antibody titers is especially helpful in determining *B. anthracis* exposure histories as antibody titers to anthrax toxin appear to not last >12 mo (Turnbull et al., 1992). Antibody detection can determine recent exposure even if the animal is not actively infected, which cannot be extrapolated from bacterial culture data alone (except in the case where a tolerant animal is positive for bacteria and has rising

TABLE 2. Comparison of available tests for anthrax exposure in wildlife populations.

Test	Method[a]	Sensitivity (%)	Specificity (%)	Advantages	Disadvantages
Antibody detection	ELISA			Measures recent infections and exposure Less expensive than ICMs Can measure antibody titers and extrapolate to time course of infection	Indirect measure of infection Most are species specific Host specific antisera not available Not commercially produced as kits for wildlife species Lack of standardization
Turnbull et al., 1986; Turnbull et al., 1992	Competitive inhibition ELISA	Varies by species tested; for humans, unknown for wildlife samples	1986: >99%	Well-described, inexpensive, widely used Available for all three components of anthrax toxin Self-describe issues about assay procedure, so limitations are known and understood	Needs much antigen Sensitivity/specificity issues for certain species Needs species-specific antiserum or uses conjugated antisera from close relatives More complex than standard ELISA Not commercially produced Lack of standardization
Immunetics	ELISA with two recombinant PA conjugates	100[b]	99[b]	Species independent, can compare AB titers across species. Rapid (45 min). Has been used successfully on six species and enabled cross-comparisons.	Product availability is questionable Only two published reports on its use Not validated for use with nonhuman animals
Peterson et al., 1993	ELISA uses bioconjugated white tailed deer sera			Single-sandwich wildlife assay Tested using vaccinated and unvaccinated white tailed deer samples	Only one published report on its use Limited contribution to knowledge about infection in wild deer Specific to deer
Fansanella et al., 2007	Unknown	Unknown	Unknown	Some information about PA antibody prevalence in red deer population	Not much known about the assay One published report on its use, no publication on its procedure

TABLE 2. Continued.

Test	Method[a]	Sensitivity (%)	Specificity (%)	Advantages	Disadvantages
Antigen/ spore detection				Active infections. Not well described in wildlife samples, environmental samples, or livestock species.	More expensive than ELISA (cost comparison) Cannot measure recently cleared infections No published data on natural wildlife samples May cross-react with other *Bacillus* spp. Lacks sensitivity
Burans et al., 1996	LFIA; detects PA	100% at 25 ng/mL; 60% at 12.5 ng/mL; 18% deer ($n=19$);[c] 0% bison ($n=16$);[c] 100% elk ($n=2$)[c]	>98% for human and livestock samples 100% for other *Bacillus* spp. 0% deer ($n=19$)[c] 37.5% bison (n=16)[c] NA elk ($n=2$)[c]	Rapid, species independent	Successful use on wildlife samples is mentioned by Burans et al. (1996) but not published. Low specificity and sensitivity in wildlife (i.e., deer and bison). Low sample size in elk ($n=2$)
BTA	LFIA; detects spores	10^6 spores: 100%; 10^5 spores: 2.5%; 10^4 spores: 0%	100	Rapid, species independent; high specificity	Reliably detects only spore loads $\geq 10^6$
BADD	LFIA; detects spores	10^5–10^6 spores: 100%; 10^4 spores: 0%	100	Rapid, species independent; reported high specificity	Reliably detects only spore loads $\geq 10^5$
SMART II	LFIA; detects spores	10^5–10^6 spores: 100%; 10^4 spores: 0%	75	Rapid, species independent	Reliably detects only spore loads $\geq 10^5$ Low specificity

[a] ELISA = enzyme-linked immunosorbent assay; LFIA = lateral flow immunochromatographic assay; PA = protective antigen.

[b] Based on clinical trials on human samples reported by the company.

[c] J. Blackburn, unpublished data.

Terms	Other nomenclature	Technical details	Adapted from
Enzyme-linked immunoabsorbent assay (ELISA)	Indirect ELISA	Assay designed to detect a protein/antigen/antibody of interest. For most disease surveillance systems, ELISAs are designed to detect a specific antibody. Commonly, a plastic plate is coated with an antigen from the pathogen of interest. If the sample contains antibody to that pathogen, it binds to the antigen on the plate. A secondary enzyme-labeled antibody is added, which binds to antigen-antibody complex, and an oxidative process produces fluorescence that is detected by spectrophotometry. Often a cutoff value is determined using a sample of known exposure history to help designate what signal levels correspond to the presence of the antibody of interest.	Crowther, 2000
Anthrax ELISA types according to Turnbull et al., 1992			
Single sandwich		Measures anthrax antibody in a traditional way: antigen + anti-anthrax antibody + substrate conjugated antibody specific to species being tested.	Turnbull et al., 1992
Double sandwich		Measures anthrax antibody in wildlife in the following fashion: antigen + anti-anthrax antibody + anti-wildlife rabbit/guinea pig antibody + anti-rabbit or guinea pig substrate-conjugated antibody.	Turnbull et al., 1992
Competitive inhibition	Inhibition; competition	An ELISA assay that includes inhibition rows to which additional measured antigen is added. Every tested sample has a matching inhibition row. The added antigen competes with the antigen bound to the plate for antigen-specific antibody in added sample sera.	Turnbull et al., 1992
Lateral flow immunochromatographic assays (LFIAs)	ICT, ICM	A handheld assay that includes a nitrocellulose membrane with two monoclonal antibodies: a signaling antibody (bound with gold colloidal particles) and an antigen-specific antibody. The sample is added to the delivery pad of the device. If the target antigen is present in the tested sample, the signaling antibody binds to the antigen of interest. This complex migrates across a nitrocellulose membrane, where it binds to the sample capture antibody and produces a readable signal (formation of an additional line or other marking) after 10–15 min of incubation.	Burans et al., 1996
Anthrax toxin		One of the two virulence factors responsible for anthrax's lethality, consists of three proteins that are not harmful on their own, but when combined in pairs or trios can cause host pathology and death.	Collier and Young, 2003
Protective antigen (PA)		Transport protein that facilitates the transport of the other two proteins (EF and LF) into the host cell cytosol.	
Edema factor (EF)		Enzyme that disrupts protein interactions in host cells, causing edema in host.	
Lethal factor (LF)		Enzyme that disrupt protein interactions in host cells, linked to host mortality.	

antibody titers). In addition to these other advantages, well-developed anthrax ELISAs do not cross-react with other *Bacillus* species (Turnbull et al., 2008).

Disadvantages: In comparison to culture, antibody detection does not directly specify current infection. At most, one can infer how recently an animal was exposed (comparison of IgG to IgM titers) and whether it is likely to be currently infected by comparing antibody titers to titers collected from an infected animal with a known exposure or infection date (as in a controlled laboratory study that measures bacterial and antibody dynamics simultaneously). One might also serial sample a host over time to see if titer levels are rising or falling, which may be difficult in free-ranging wildlife. Also, most traditional anthrax ELISAs use species-specific reagents (Turnbull et al., 1986; Turnbull et al., 1992; Peterson et al., 1993; Fasanella et al., 2007). In the case of humans, livestock, or single host wildlife systems, using a species-specific ELISA is less problematic (Peterson et al., 1993). However, in many anthrax zones, surveillance involves multiple host species, as *B. anthracis* has a wide host range. For example, in African national parks, antibody to anthrax was detected in up to six species, including herbivores, their scavengers, and predators (Turnbull et al., 1992; Hampson et al., 2011; Lembo et al., 2011; Table 1). Multiple host surveillance can be problematic, because species-specific reagents are generally not commercially available for wildlife, and improvisation in assay reagents is often necessary. Historically, researchers produced antisera in-house and used "double sandwich" competitive inhibition indirect ELISA assays (Turnbull et al., 1992; see Glossary) or utilized a "single sandwich" competitive inhibition indirect ELISA (Turnbull et al., 2004; Good et al., 2008; Turnbull et al., 2010) with commercially available conjugated antisera of the closest relatives of the wildlife species. Non–species-specific antisera often leads to diminished sensitivity and specificity of the assay when used on wildlife samples. This effect can be difficult to measure in wildlife in the absence of comparisons between vaccinated and unvaccinated animals or animals with known exposure histories (Turnbull et al., 1992; Turnbull et al., 2010). Most anthrax ELISAs used on wildlife have not been formally validated for the tested species, and all but one assay (Immunetics, Boston, Massachusetts, USA) are not commercially available.

Of four published indirect ELISAs used on wildlife samples, one was used extensively on wildlife populations, the competitive inhibition indirect ELISA assay (Turnbull et al., 1986). The QuickELISA Anthrax PA Kit (Immunetics; Lembo et al, 2011) was used in two reports of serosurveillance in wildlife populations. The other assays are specific to white-tailed deer and red deer (*C. elaphus;* Fasanella et al., 2007), each having one published report (Table 2).

The Turnbull ELISA, or noncompetitive variations of it, is the first and most widely used serologic screening approach for anthrax exposure in animals (Turnbull et al., 1986, 1989, 1992, 2004, 2010; Rijks, 1999; Good et al., 2008; Bellan et al., 2012). It was used with sera collected from multiple carnivores and herbivores across parts of Africa, domestic swine from Wales, and bison from Canada (Table 1). Most knowledge about wildlife anthrax exposure emanated from this procedure or its modifications. There are also Turnbull ELISAs to detect antibodies to EF and LF. As the Turnbull ELISA is not commercially available, researchers often re-establish this assay in their laboratory and need to re-evaluate and validate the test for the wildlife species of interest.

The Immunetics QuickELISA uses recombinant technology to detect PA antibody independently of Ig-subtype and host species. This is a promising approach for anthrax surveillance, particularly as the assay allows cross-species

comparisons. However, the assay was designed for use on human samples, and wildlife testing is an off-label use. As stated previously, there are only two published reports using the Immunetics assay, both focused on wildlife within the Serengeti ecosystem, Tanzania (Hampson et al., 2011; Lembo et al., 2011). All serologic methods for anthrax have poorly understood sensitivity and specificity in wildlife. Frequently it is difficult to ascertain the exposure history of free-ranging wildlife, and the availability of positive and negative controls of exotic species is nearly impossible, unless there is a captive population with a known exposure history available.

Lateral flow immunochromatographic assay

Advantages: The LFIA is a rapid, handheld assay format that detects PA in blood or serum. LFIAs are a direct measure of exposure; they can be used to detect PA circulating in the blood, which indicates the animal is actively infected or potentially has absorbed PA after consuming an anthrax-laden carcass. These assays have a rapid turnaround time (~15 min) and theoretically work for multiple species.

Disadvantages: The LFIA is limited as a surveillance tool for measuring background exposure, because animals must be circulating PA antigen. Limited evidence suggests other components in blood samples may interfere with LFIA test results. Although theoretically an efficient way of detecting current infection in wildlife, they apparently lack sensitivity (and specificity with some antigens). This can be problematic, because PA can degrade below detectable levels relatively quickly (≤48 hr; Muller et al., 2004), making these kits most effective immediately postmortem. Spore kits were designed for environmental detection (e.g., surfaces and water) and may not be as useful at carcass sites. Published data suggest none of the available spore detection assays were able to detect spore loads below 10^5 spores, limiting their use in detection of the pathogen associated with wildlife. Animal carcasses are also likely to be contaminated with other *Bacillus* species that may cause false positive results.

Four LFIAs have a published review of their efficacy (spore LFIAs; King et al., 2003) or a publication regarding their use for serum samples (Muller et al., 2004). One LFIA detects PA (Burans et al., 1996), while the other three are commercially available spore protein detection LFIAs (Anthrax Biothreat Alert test strips [Tetracore, Gaithersburg, Maryland, USA]; BioWarfare Agent Detection Devices [Osbourne Scientific, Lakeside, Arizona, USA]; and Anthrax SMART II [New Horizons Diagnostics, Columbia, Maryland, USA]).

The Burans et al. (1996) PA assay was reported to have high specificity (>98%) with negative human, livestock, and wildlife sera (Muller et al., 2004), as well as in sera spiked with related *Bacillus* species other than *B. anthracis* (Burans et al., 1996; Table 2). However, data using this LFIA on wildlife samples have not been published. Also, starting in the early 2000s, this LFIA was field tested across wildlife and livestock outbreaks in Texas and Canada as postmortem, "carcass-side" tests. Based on results from outbreaks in white-tailed deer in 2005–2007 in Texas and bison from Canada in 2007, sensitivity was low in both species across a range of carcass conditions (e.g., time past death; Blackburn, unpubl. data; Table 2). This assay is not commercially available and has not been formally validated for use on animal samples.

Spore detection LFIAs have reported detection targets of 10^4–10^5 spores per sample. However, a recent performance assessment showed only two of three assays reliably detected 10^5 *B. anthracis* spores, and one gave a positive result when exposed to another *Bacillus* species (King et al., 2003; Table 2). There are no published reports of the use of this assay with wildlife samples. One study employed New Horizon kits with swab samples from wildlife carcasses (e.g.,

hides, bone swipes) during bison outbreaks in Canada and white-tailed deer outbreaks in Texas in 2007 and had no positive tests (Blackburn, unpubl. data).

Recommendations

Surveillance assay selection: Available ELISAs should be used for determining host exposure to *B. anthracis* in living animals. Current LFIA tests should be used as carcass-side postmortem tests in freshly dead animals and matched with gold standard pathogen tests. Assay sensitivity and specificity should be determined before use with the animal sera as matrix.

Reliable serological assays: To further advance anthrax serosurveillance, reliable assays are needed. Commercially available wildlife serologic assays would be ideal. Well-validated reagents and species specific antibodies are crucial, as are reliable assays that allow cross-species comparisons.

Multiple assay approaches: Although bacterial culture and PCR continue to be the gold standard diagnostics for anthrax, they have not been used in conjunction with serologic methods in wildlife species. Examining the pathogen (culture, PCR, LFIA) and the host immune response (ELISA) and the possible interplay between them is essential to further understand the progression of infection in hosts and may enable some novel cross-species comparisons.

Investigation of other hosts and ecosystems: The majority of anthrax serosurveys have been conducted in African wildlife parks. Serosurveillance is needed in other enzootic anthrax zones. These zones represent different ecosystems and ecological factors influencing anthrax occurrence and environmental spillover. Obtaining data from herbivores, carnivores, and omnivores simultaneously will improve our understanding of exposure patterns, infection risk, disease resistance, and survival in wildlife species.

Expand use of sentinel species: Vultures, and possibly other scavengers (e.g., coyotes, *Canis latrans*; Mongoh et al., 2008), may be ideal sentinel species to better determine the geographic extent of anthrax, as they often cover large geographic areas.

While postmortem surveillance provides information about the ecology and location of *B. anthracis*, it cannot, alone, provide a complete picture. Increased antemortem serosurveillance is critical to increase our understanding of exposure risk and the geographic extent of anthrax in wildlife. To achieve this, more standardized and well-developed serologic anthrax detection assays are needed, along with efforts to deploy these tools across areas of known anthrax risk.

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